Preprint
UCRL-JC-143868

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This article was submitted to
North Atlantic Treaty Organization Symposium on Operational Medical Issues in Chemical and Biological Defense, Lisbon, Portugal, May 14-17, 2001

U.S. Department of Energy

Lawrence
Livermore
National
Laboratory

May 14, 2001

HFM SYMPOSIUM
ON
"OPERATIONAL MEDICAL ISSUES IN CHEMICAL AND BIOLOGICAL DEFENSE"

DETECTION OF PATHOGENS USING A HAND-HELD PCR THERMOCYCLER

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International concern about vulnerability to biological weapons of mass destruction has prompted the development of technologies to address shortfalls in on-site detection. Recent advances in thermocycler technology have allowed the miniaturization of a pathogen detector based on real-time fluorogenic polymerase chain reaction (PCR). PCR can detect minute quantities of DNA from living organisms associated with biological weapons.

PCR is essentially a DNA amplification reaction in which oligonucleotide primers are designed to be specific for a particular gene of the target pathogen. The primers anneal to the DNA of the pathogen and the PCR thermocycler is repeatedly cycled to create copies of the DNA in an exponential fashion. Real-time fluorogenic PCR is a variation of traditional PCR in which an optical signal is produced that allows the user to view the accumulation of the specific DNA through out the course of the cycling. Because of the high degree of amplification possible in PCR, the thermocycling detector is capable of detecting as little as a single bacteria or virus. Even crude preparations of toxin can have residual DNA associated with them that can also be detected, making this technique a sensitive complement to antibody-based detection schemes.

One of the obstacles to putting this technology into the hands of first responders or soldiers is the size of commercially available instrumentation. For example

the Applied Biosystems 7700, while exquisitely sensitive, weighs 320 pounds (146 kilograms). Researchers at the Lawrence Livermore National Laboratory (LLNL) have created a miniature man-portable PCR thermocycler/detector called the HANAA (hand-held advanced nucleic acid analyzer). The HANAA is the culmination of six years of PCR instrument development at LLNL, starting in about 1994 and was first publicly demonstrated at IC Demo 99, in September and October of 1999. The unit is currently being beta tested at ten sites, is entering commercialization by Environmental Technologies Group, Inc, and will be ready for sale in 2002. The operator interface is menu-driven and controlled using a simple 16 key keypad. This enables the user to step through the on board programs for set-up and operation of the assays. The menu and run information are displayed on a 2-inch LCD display. Once a run is initiated, thermal cycling is controlled automatically. Amplification and detection are indicated by easy to understand bar graphs for each sample, along with an audible alarm and color change for detection of any positive samples. The PCR data are stored on board and can be downloaded to an external computer for more complete analysis.

The introduction of this unit opens up the possibility of placing PCR technology into the hands of the military combat and reconnaissance personnel and first responders in the domestic preparedness field where large cumbersome machines are too costly or too bulky to be effective. Whether the HANAA will be effective for these users will depend on the sensitivity and reliability of the Taqman PCR assays that are directed towards biological pathogens of concern.

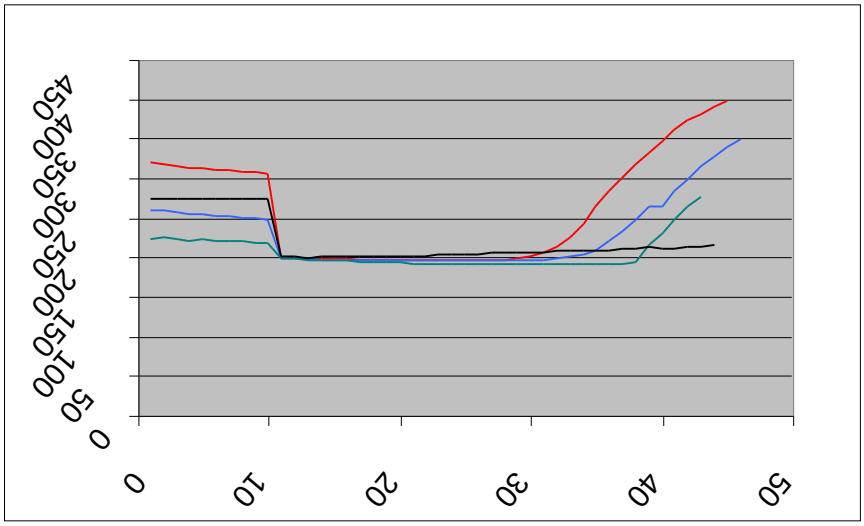
The heart of the HANAA is the sample module that houses the unique fluorescent detection system and LLNL's advanced micromachined silicon thermal cycler. The current HANAA prototype with a sample module removed is shown in the figure. The thermal cycler incorporates anisotropically etched features that greatly reduce the thermal mass and create added surface area and integrated passages to enhance forced air-cooling. The instrument is controlled by two microcontrollers and an embedded 386 microprocessor.

Taqman assays rely upon the intrinsic exonuclease capability of the enzyme used to amplify DNA in a PCR reaction. The enzyme, Taq DNA polymerase, degrades DNA that anneals in its path as it is synthesizing new DNA. PCR reactions exponentially amplify DNA through the use of a forward and a reverse DNA oligonucleotide primer. If a small DNA probe anneals to the target gene in between a forward and a reverse DNA primer, the Taq DNA polymerase will degrade that DNA as it "rolls over" it. Taqman chemistry makes use of that phenomenon by placing a fluorogenic emitter on one end of the probe DNA and a fluorogenic quencher on the other. When the quencher and the emitter are bound to the DNA probe there is little emitted fluorescence. As Taq DNA polymerase chews through the probe, the quencher and the emitter are separated allowing light to be emitted. As the PCR reaction proceeds through several rounds, more and more fluorescence is released as the DNA is amplified, and eventually the signal crosses the instrument optical detection threshold, registering an alarm. HANAA has two detection systems on each of it's four chambers, one with a blue LED optimized for detecting the FAM dye and the other with a green LED optimized for the JOE or TAMRA dyes.

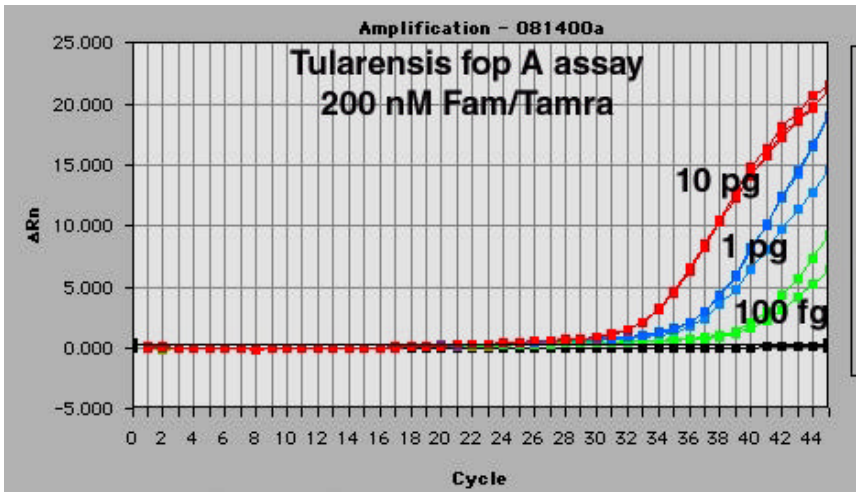
Edgewood Chemical Biological Center and the Armed Forced Institute of Pathology have developed Taqman PCR assays for a variety of biological agents. These assays were optimized for use on stationary platforms such as the Applied Biosystems 7700 sequence detection system and the Idaho Lightcycler. These PCR systems are rapid thermocyclers that are difficult to field due to their size, power consumption, and sensitive optics. The PCR assays can be adapted to any properly configured optical PCR thermocycler although the formulations used for the reaction mix must be adjusted.

The HANAA unit is small and measures 4 by 10 by 2 inches. It weighs a mere 2 pounds and has a 2.5 pound battery capable of running the instrument for 1.4 hours. That time is sufficient for at least three complete runs using all four chambers and will allow for the analysis of 12 samples with two measurements on each smple. The addition of an optional 11 pound battery belt can extend the running time to 5.5 hours. The system is durable and easy to use, and the 25 microliter plastic sample vials are easy to load.

The HANAA unit was tested by taking Taqman assays that had been optimized on the ABI 7700 unit and transitioning them onto the HANAA. The ABI 7700 can be set to run 25 microliter volumes to mimic the HANAA volume. Primer ratios and probes are optimized on the ABI 7700 because it has a 96 well capacity which is ideal for high throughput testing such as this. One of the shortcomings of the four-chambered HANAA is that development is time consuming. The introduction of the 24-chambered PolyHANAA should speed up some of that assay optimization work, now that it is completed. There was a need to replace the buffer salts that were commonly used on the ABI 7700 since the unit uses ROX dye as an internal standard for providing a baseline and this caused a higher background in the HANAA.



Once it was shown that we could transition assays from the ABI 7700 to the HANAA with slight modifications we sought to determine the sensitivity of the two units using the same assay. The fop A gene of *Francisella tularensis* is an outer membrane protein that serves as a sensitive Taqman PCR assay target. Purified genomic DNA from *F. tularensis* SCHUE 4 strain was added at 10 picograms, 1 picogram, and 100 femtograms and run in both the ABI 7700 and the HANAA. Similar sensitivity was observed in both units that can be extrapolated to a detection threshold of approximately 10 bacteria.



Threshold values (C_T) are often used to compare assay performance and the thermocycler software interprets the threshold value (C_T) to be the point at which the ratio of the reporter (FAM) to the quencher (TAMRA) exceeds a set limit and the amplification reaction is considered to be statistically significant. The C_T is often 10 times the standard deviation of the baseline, or it can be set just slightly above the negative controls to compensate for degradation of the probe over the course of the run. The threshold at which the fop A assay registers a positive response for a given concentration of DNA is similar for both instruments. The key difference is that the ABI7700 takes three hours to run the experiment and the HANAA takes 27 minutes. Furthermore the results from the HANAA are updated throughout the course of the run and can be viewed on a bar chart that indicates when a sample has breached the threshold. The ABI7700 must complete the three hour run and then the samples are processed through an analysis program which can be interpreted using programmed algorithms.

Speed is often a criterion when biological weapons are being analyzed and a quick turn around in the analysis can have profound implications. The speed, real-time analysis capability, and portability put the HANAA in a class by itself among PCR based detectors. The completion of the beta testing and the introduction of the HANAA2 for commercial sale should provide a welcome tool for the first responder. Commercialization and large volume production should lower the cost of the unit to a few thousand dollars and create a large market for freeze-dried Taqman PCR assays for weapons of mass destruction. We look forward to the introduction of field worthy, hand held nucleic acid detectors that can expand our current capabilities and believe that this device shows great promise.

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*This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract No. W-7405-Eng-48.